ORGANIZATION AND COMPOSITION OF THE AMPHIBIAN YOLK PLATELET. II. INVESTIGATIONS ON YOLK PROTEINS[1]

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As indicated elsewhere (Ringle and Gross, 1962), an understanding of the organization and composition of yolk platelets is a prerequisite for an adequate appreciation of the factors involved in yolk synthesis and utilization. Since amphibian yolk platelets are primarily lipo- and phosphoprotein structures, information pertaining to the composition and behavior of the protein components is important for understanding the make-up of the intact platelet.

The results reported here include investigations on both the soluble and insoluble fractions derived from lysed platelet suspensions of *Rana pipiens*. These fractions were studied by a number of chemical and immunological methods.

MATERIALS AND METHODS

Washed yolk platelets were prepared from ovarian eggs of *Rana pipiens* by the homogenization-centrifugation method of Essner (1954) as previously described (Ringle and Gross, 1962).

1. *Solubilization of yolk platelet proteins*

Yolk platelets were lysed by lytic concentrations of monovalent or divalent cation salts, usually 0.4–0.5 *M* NaCl or 0.5 *M* $CaCl_2$. The insoluble residue ("ghost" material) of washed platelet suspensions after lysis was removed by centrifugation at 700 × *g*. Precipitation of solubilized yolk proteins was accomplished either by direct dilution with or by dialysis against hypotonic solutions (dilute saline or distilled water). Precipitates and supernatants were separated by centrifugation. A summary of the soluble, insoluble, and precipitate fractions derived from washed platelet suspensions is given in Figure 1.

2. *Extraction of lipids*

NaCl-solubilized yolk (YP_{na}) in 0.5 *M* NaCl was lipid-extracted by a method similar to one described by Macheboeuf (1953) for the extraction of lipids from plasma lipoproteins. YP_{na} was lipid-extracted with an equal volume of ethanol: ether (9.6% ethanol by volume) by vigorously shaking 50–80-ml. volumes of YP_{na} with ethanol: ether in stoppered separatory funnels. These solutions were thus

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shaken for 20–30 seconds three times at approximately 30-minute intervals. Following extraction the saline (containing lipid-extracted yolk, YP_{df}) and ether fractions were separated and refrigerated for future analysis.

Carotenoids from the ether fraction were characterized by the analytical method described by Fox (1953) for the separation of carotenes and xanthophylls. The separation of these carotenoids is based on their differential solubilities in petroleum ether and in 90% methanol. Photometric analyses (optical density readings) of the carotenoids dissolved in petroleum ether were performed with a Beckman DU model 2400 spectrophotometer.

3. *Electrophoresis*

Precipitated soluble yolk fractions YP, YC, WC and YP_{df} (for the method of preparation see Results, 1.) were dissolved in pH 10.1 glycine-NaOH buffer and separately dialyzed under refrigeration for 24–48 hours against one liter of the buffer used for solubilization. Portions of these buffer-yolk solutions were refrigerated in stoppered containers and conductivity measurements performed 5–9 weeks later with a Leeds and Northrup conductivity bridge. Conductivity measurements showed almost identical conductivities for the stored solutions but, since these solutions had been stored, the conductivity values were not used to calculate protein mobilities. However, the similarity of the conductivity values made it reasonable to compare the number, size and distribution of peaks among the various runs. Electrophoretic analyses were performed with a Perkin-Elmer Model 38A electrophoresis apparatus equipped with a Rayleigh fringe accessory. Runs were conducted at 1.5° C. and 7.5 ±0.5 milliamperes.

4. *Nitrogen and phosphorus determinations*

a. Total nitrogen

Total nitrogen values were determined by the Kjeldahl method, using an Aminco-Koegel rotary digestion apparatus and an Aminco-Koegel glass distillation apparatus. Digestions were performed in dual-purpose flasks for 4 or more hours with concentrated H_2SO_4 plus selenium catalyst. Digested samples plus 5 ml. of 50% NaOH and 7 ml. of distilled water were distilled for 20 minutes. The distillate was collected in 10 ml. of 2% boric acid containing 8–9 drops of indicator (a 2:1 mixture of 0.1% bromocresol green and methyl red solutions). Titrations were done with 0.0098 N HCl. All samples were run in duplicate.

b. Total phosphorus

The procedure used was modified from methods described by Hawk, Oser and Summerson (1954) and Leloir and Cardini (1957). Digestions were performed in 30-ml. Kjeldahl flasks heated on an Aminco-Koegel rotary digestion apparatus. Flasks containing 0.5–1.0 ml. of test material and 1.0 ml. of 5 N H_2SO_4 were heated for 70 minutes. After cooling, two drops of 30% hydrogen peroxide (Superoxol) were added and the flasks heated for 20 minutes. Several milliliters of distilled water were then added and the flasks again heated for 20 minutes. After cooling 1.0 ml. of 2.5% ammonium molybdate was added to each flask and the contents then quantitatively transferred to graduated 12-ml. conical centrifuge tubes with three distilled water washes. Additional distilled water was added to

bring the tube contents up to 9.8 ml. Reducing solution was prepared shortly before use by dissolving, in 10 ml. of distilled water, 250 mg. of a stock dry mix composed of 0.2 gm. 1-amino-2-naphthol-4-sulfonic acid, 1.2 gm. sodium bisulfite and 1.2 gm. sodium sulfite. To each tube was added 0.2 ml. of reducing solution and, 10 minutes after mixing, optical density values read at 660 mμ with a Bausch and Lomb Spectronic "20" spectrophotometer. The sample phosphorus content (μM P/sample) was determined from the o.d. readings by reference to a standard curve. All samples were run in duplicate.

5. *Nucleic acid determinations*

DNA (deoxyribonucleic acid) and PNA (pentose nucleic acid) were extracted from "ghost" and yolk samples with hot trichloroacetic acid by the method described by Schneider (1957). Most extractions were performed in 12-ml. conical glass centrifuge tubes, although in some cases 40-ml. tubes were used to accommodate larger samples (for which proportionately larger volumes of reagents were used for extraction). DNA-P and PNA-P in the extracts were determined using diphenylamine and orcinol reagents as described by Schneider. Although DNA and PNA standards were run and standard curves plotted, the values reported here were calculated for nucleic acid phosphorus by the formula given by Schneider. DNA-P and PNA-P values were calculated from the averaged o.d. readings of duplicate runs.

6. *Antigenic analyses*

a. Production of antisera

Soluble yolk antigen and "ghost" antigen were prepared by lysing yolk platelet suspensions with 0.4 M NaCl or with 0.5 M $CaCl_2$ + 0.1 M NaCl. "Ghost" material was separated from the soluble yolk fraction by centrifugation and washed 3–4 times in lytic salt solutions to remove soluble yolk. Soluble yolk and insoluble "ghost" fractions were stored separately under refrigeration prior to their injection as antigens. Protein concentrations of the antigen solutions were estimated from the volume of packed platelet material used for lysis (by the biuret method it was found that one ml. of packed platelets yields about 375 mg. of soluble protein). To produce antisera for the tube double diffusion tests, the protein concentration was approximately 28 mg. per ml. whereas a protein concentration of about 10 mg. per ml. was used to produce antisera for the Petri dish double diffusion test. "Ghost" protein concentration was one mg. per ml. Antigens were injected intravenously, one ml. per injection, with a total of 5–8 injections per animal, spaced 3–4 days apart. Albino rabbits (mixed sexes) and New Hampshire Red roosters were used. Injection, bleeding (intracardiac puncture), and serum preparation techniques were as described by Kabat and Mayer (1948). Sera were either used immediately after preparation or were shell-frozen in vials in an acetone-dry ice mixture and stored in a freezer for future use.

b. Tube double diffusion method

Antigen-antibody analyses in tubes were done in rimless culture tubes (10 × 75 mm.) which had been internally coated with a thin layer of agar as described by Munoz and Becker (1950). Solutions were introduced into these

tubes with hand-drawn glass pipettes fitted to one- or two-ml. syringes by means of surgical rubber couplings. Serum (0.75 ml.) was overlayed with 2.0 ml. of warm (50–55° C.) 1% agar (Difco). After a brief refrigeration to hasten solidification of the agar, 0.75 ml. of antigen was added over the agar. The tubes were tightly stoppered with corks and stored at room temperature. Tubes were examined every other day for band formation (*i.e.*, antigen-antibody precipitates in the agar column).

All solutions used (agar, serum and antigen) contained 10 μg. of chloromycetin per ml. Other substances added are indicated in Table IV. Agar pH was adjusted to 7.0 ± 0.1 with 0.2 N NaOH.

c. Petri dish method

Petri dishes for the Ouchterlony method were filled with 75 ml. of 1% agar and refrigerated briefly. Wells to contain the reactants were made by forcing three 19-mm. diameter glass cylinders into the agar bed and were spaced 2 cm. apart. The agar within each cylinder was removed with a medicine dropper, leaving a thin layer of agar at the bottom of each well. Sera or antigen solutions were added to each well, the glass cylinders withdrawn, and the completed dishes covered and stored under a bell jar to reduce evaporation. All reagents used contained 1 : 10,000 Merthiolate. Agar pH was initially adjusted to 7.0 with 0.2 N NaOH and was finally made up in 0.05 *M* Tris buffer (pH 7.2) containing 2.0 *M* NaCl and 0.002% methyl orange. Sera contained 2.0 *M* NaCl and antigen solutions were either in 0.4 *M* NaCl or 0.05 *M* $CaCl_2$ (see Figure 4).

Results

1. *Solubility of yolk platelets and yolk "fractions"*

The studies on the solubility of yolk platelets and yolk "fractions" precipitated from solubilized yolk are summarized in Figure 1.

a. Solubility in $CaCl_2$

Yolk platelet suspensions were lysed in 0.05 *M* $CaCl_2$, although both higher and lower concentrations of this salt will also result in platelet lysis (Holtfreter, 1946; Terry, 1950; Essner, 1954; Gross, 1954). Lysis in 0.05 *M* $CaCl_2$ resulted in a rapid dissolution of soluble yolk, leaving behind an insoluble "ghost" material which could be readily removed by low speed centrifugation. The "ghost"-free calcium-lysed yolk (YP_{ca}) was not as optically clear as NaCl-lysed yolk (YP_{na}), as has been previously reported (Gross and Gilbert, 1956). This mild turbidity was also noted in NaCl-lysed yolk solutions containing 0.05 *M* $CaCl_2$. No visible reduction in turbidity followed centrifugation at 41,150–56,500 × g for 15 minutes, thereby indicating that the turbidity was caused by particles less than 1000 Å in diameter. YP_{ca} was more easily surface denatured than YP_{na}, the former readily yielding stringy precipitates upon shaking or stirring. Also, refrigeration of YP_{ca} for 1–2 days was accompanied by a spontaneous precipitation of some flocculent material which was not noted in similarly treated YP_{na}.

Although prolonged exposure to calcium ions causes amphibian yolk to become permanently soluble, dilution with or dialysis against distilled water soon after

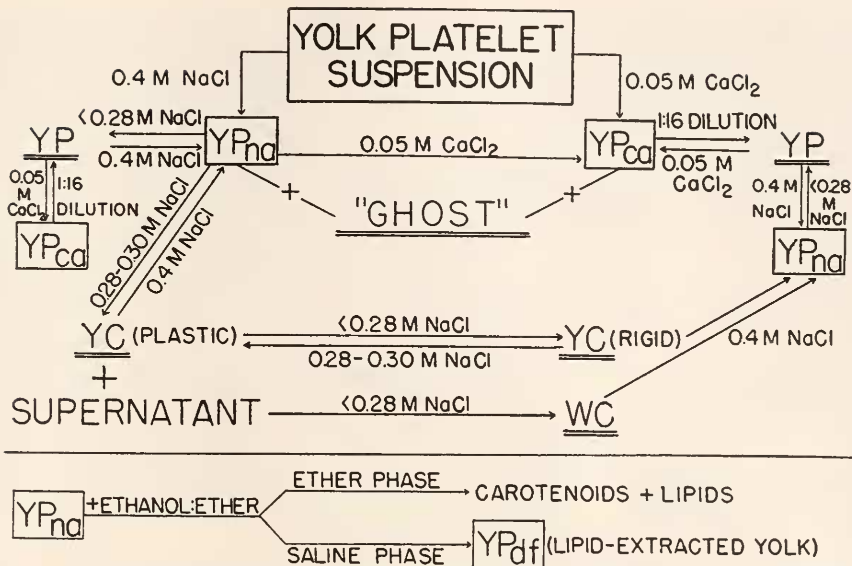

FIGURE 1. Soluble and insoluble fractions derived from washed yolk platelet suspensions. Detrital "ghost" material formed from both NaCl- and $CaCl_2$-lysed suspensions.

lysis promotes yolk precipitation (Flickinger, 1956). This finding was confirmed in the studies reported here. Both freshly lysed and refrigerated (up to three hours or more) YP_{ca} in 0.05 *M* $CaCl_2$ could be readily precipitated by 1 : 16 dilution with distilled water (one part YP_{ca} + 15 parts distilled water). Dilution 1 : 8 did not cause a precipitation of the solubilized yolk, thus indicating the critical concentration of $CaCl_2$ required for solubilization of *Rana pipiens* yolk to be near 0.003 *M*.

There was a marked difference in the kinetics of precipitation of soluble yolk (YP) from YP_{na} and YP_{ca} solutions: dilution of YP_{na} to a low salt concentration (0.2 *M* or less NaCl) resulted in a rapid precipitation of all the YP, whereas 1 : 16 dilution of YP_{ca} in 0.05 *M* $CaCl_2$ showed several seconds delay before the initial appearance of YP. Moreover, YP continued to accumulate for a minute or more after the 1 : 16 dilution. Precipitation seemed to be complete in 30 minutes or less, as judged by the failure of the resulting supernatant to yield further precipitate either by further dilution with distilled water or by the addition of an equal volume of 10% TCA.

b. Fractional precipitation of NaCl-lysed yolk (YP_{na})

Lysis of washed yolk platelet suspensions in 0.4 *M* or higher concentrations of NaCl yielded optically clear, yellow YP_{na} and an insoluble "ghost" material readily packed by centrifugation. This "ghost" fraction was similar in appearance to the "ghost" fraction resulting from lysis by $CaCl_2$. As previously reported (Ringle and Gross, 1962), the "ghost" fraction was found to consist largely of detrital remnants of ovarian and follicle membranes, together with some insoluble platelet material.

Precipitation of yolk from YP_{na} by dilution gave either of two physically different types of precipitate, depending upon the salt concentration of the medium in which the precipitation occurred. At salinities below 0.28 *M* NaCl a light, flocculent, creamy-white material (YP) formed; at 0.28–0.30 *M* NaCl there appeared a dense, viscous precipitate (YC). A proportionately small quantity of YP-like precipitate (WC) formed upon further dilution of the supernatant from YC preparations. At room temperature the dense YC material was yellow and transparent, much resembling honey in both appearance and fluidity. At low temperatures (3–5° C.) YC became opaque and rigid (Ringle, in press). YC also became opaque and rigid at room temperature if transferred to NaCl concentrations below 0.28 *M*, resulting in the formation of a dense material which could be broken up to yield irregular platelet-like YC-fragments. These YC-fragments behaved like platelets in lysis and micromanipulation experiments, and they also showed a relatively orderly and dense packing of yolk components as revealed by electron microscopy (Ringle and Gross, 1962). YC material occupied the same volume as the packed yolk platelets used to produce it (Table I).

After the precipitation of YP or WC material, no detectable proteins remained in solution, as indicated by the failure of further precipitate to form upon dilution of the supernatant with an equal volume of 10% TCA.

Although YP and YC were markedly different in appearance, they behaved similarly in lytic salt solutions. Furthermore, either YP or YC could be precipitated from redissolved YP or YC by reduction of the salt concentration to the proper level (0.28–0.30 *M* NaCl for YC, below 0.28 *M* for YP). YP precipitated from lysed yolk in 0.05 *M* $CaCl_2$ by 1:16 dilution with water could also be converted to either YC or YP by re-lysing in NaCl and subsequent dilution to the proper salt concentration. As indicated below, YP- and YC-type precipitates could also be produced from lipid-extracted YP_{na}.

2. *Extraction of lipids from YP_{na}*

Extraction of lipids from NaCl-lysed yolk by 9.6% ethanol:ether removed about 10% (9.0–11.1%) of the dry weight of YP. The extracted lipid contained all or most of the carotenoid pigment of yolk, since lipid-extracted yolk protein was white in color and did not form yellow solutions when dissolved in saline. Carotenoid pigment was largely xanthophyllic, since practically all of the yellow

TABLE I

*Volumetric analysis of yolk platelet suspension and its derivative YC, WC and "ghost"**

Item	Volume (ml.)
1. Washed yolk platelet suspension	4.90
2. YC (in 0.3 *M* NaCl)	4.00**
3. "Ghost" material	0.75
4. WC (in 0.1 *M* NaCl)	0.05
Total of items 2, 3 and 4	4.80

* Materials packed in graduated tube by centrifugation.

** This value slightly lower than its true value because a small portion of the sticky YC could not be removed from the dialysis tubing.

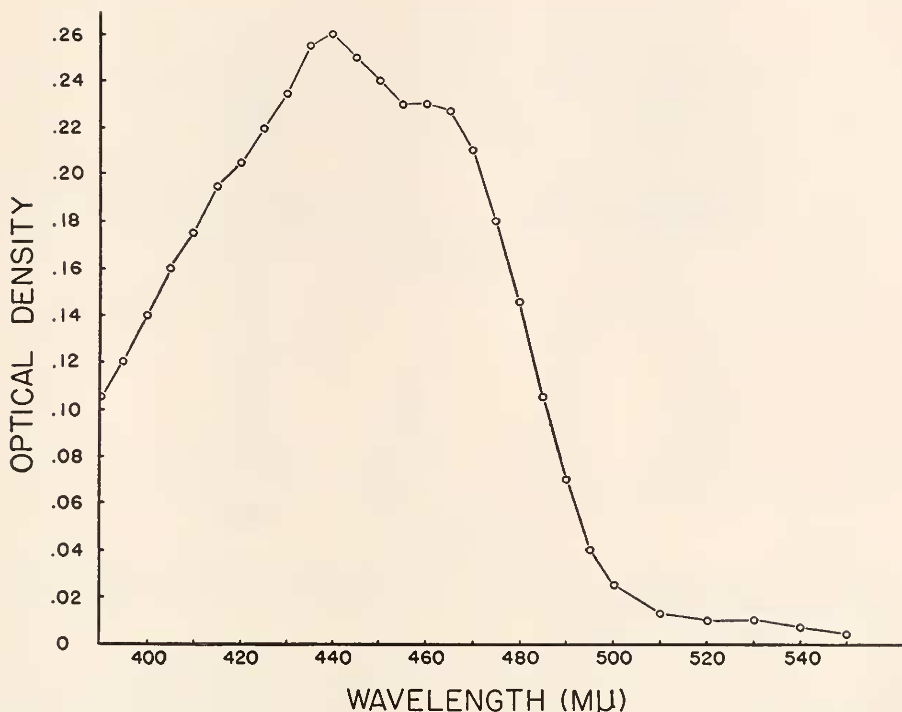

FIGURE 2. Absorption spectrum of yolk xanthophylls in petroleum ether. Absorption maximum at 440 mμ.

pigment was preferentially soluble in 90% methanol with only a small portion remaining in the petroleum ether epiphase. Photometric analysis of the methanol hypophase carotenoids redissolved in petroleum ether showed a light absorption maximum near 440 mμ (see Figure 2).

Except for a small gel-like fraction (presumably denatured yolk protein) which collected at the saline-ethanol: ether interface, most of the lipid-extracted YP_{na} material (YP_{df}) remained soluble in lytic concentrations of NaCl. YP_{df} retained the solubility properties of YP_{na} and, upon dilution, could be precipitated as YP- or YC-like precipitates at the proper salt concentrations. However, the YC-like precipitate in 0.3 *M* NaCl, although dense and viscous, was not yellow and transparent like normal YC. Also, it was easily scraped or poured from dialysis tubing, whereas YC was very sticky.

3. *Electrophoresis*

As indicated in Figure 3, all the yolk fractions tested exhibited two main electrophoretic peaks: a smaller, faster component (A) and a larger, slower component (B). The descending patterns showed in addition a variable number of minor peaks which may represent additional electrophoretic species. The trailing margin of the larger B component of YP_{df} showed some asymmetry, which suggests

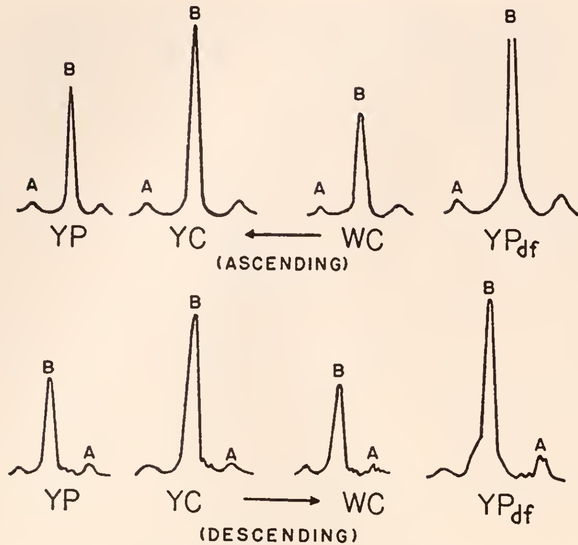

FIGURE 3. Electrophoretic patterns of soluble yolk fractions. Faster A and slower B components present in all fractions tested. Slow trailing peak (not labeled) an artifact and does not represent a yolk component.

that this peak was made up of at least two fractions. The stationary peak trailing the B peak in all runs is an artifact and does not represent an electrophoretic component.

4. *Phosphorus/nitrogen ratios*

Phosphorus/nitrogen (P/N) ratios as well as some dry weight/nitrogen ratios are given in Table II. Whole washed platelet suspension, YP (with one exception), and WC gave identical P/N ratios of 0.14. The P/N ratios for a single batch of YC and YP were slightly lower (0.13), and values as low as 0.093 were obtained for lipid-extracted YP (YP_{df}). "Ghost" material gave much higher P/N ratios, 0.20–0.21. Aging of yolk platelets and YP under refrigeration (up to 21 days), and dialysis of YP against refrigerated distilled water for 4 days, did not alter yolk P/N ratios.

Nitrogen determinations on the total "ghost" and soluble yolk portions of three different batches of washed yolk platelets showed that the "ghost" fraction represented a fairly constant proportion of the total washed preparation (Table III). This finding indicates that the homogenization-centrifugation technique for isolating yolk may be relied on to produce similar yolk preparations from different batches of *Rana pipiens* ovaries.

5. *Nucleic acids*

Nucleic acid determinations for various yolk and "ghost" samples are summarized in Table II as nucleic acid phosphorus/nitrogen ratios. "Ghost" material gave the highest DNA-P/N (0.011–0.026) and PNA-P/N (0.0038–0.012) ratios.

TABLE II

Chemical analyses on yolk platelets and yolk fractions

Item	Phosphorus/nitrogen (P/N) ratio	Dry wt./nitrogen ratio	DNA-P/N ratio	PNA-P/N ratio
Yolk platelet suspension	0.14 (2)*	—	0.00074	0.00062
YP	0.13 (1) 0.14 (4)	6.7	0– 0.00095	0.00016– 0.00072
YP_{df}	0.093– 0.12 (3)	6.4– 6.5	— —	— —
YC	0.13– 0.14 (2)	—	—	—
WC	0.14 (1)	—	—	—
"Ghost"	0.20– 0.21 (3)	6.3– 6.6	0.011– 0.026	0.0038– 0.012
Supernatant**	—	—	0.00037	0.0017

* Numbers in parentheses ( ) indicate the number of batches of yolk analyzed.
** The supernatant from the first centrifugation after ovarian homogenization.

Lower ratios were found for whole washed platelet suspension, YP_{na}, YP precipitate, and the supernatant from the ovarian homogenate.

6. *Antigenic analyses*

Antigen-antibody analyses on yolk by the double diffusion method utilized a number of different salt, antigen and antibody concentrations. Some of the results of these investigations are summarized in Table IV (tube double diffusion method) and in Figure 4 (Petri dish method). The maximum number of bands of antigen-antibody precipitate found for solubilized yolk (both NaCl- and $CaCl_2$-lysed yolk) was five. At least three of the soluble antigens of yolk were also found in "ghost" material, since antisera to "ghost" material showed three zones of precipitate when reacted with soluble yolk.

TABLE III

Total nitrogen of insoluble "ghost" and soluble fractions of washed yolk platelet preparations

Preparation	"Ghost" N (mg.)	Yolk N (mg.)	Per cent "ghost" N
A	0.90	310	0.29
B	1.60	560	0.29
C	0.78	260	0.30

TABLE IV

Antigenic analysis of amphibian yolk by double diffusion in agar

Tube #	Antigens	Concentration of saline in agar	Antisera*	Maximum # of bands
1	Calcium-lysed YP in 0.4 *M* NaCl (protein conc. 0.7%)	0.4 *M* NaCl	A-1 (0.4 *M* NaCl)	5
2	Same as above	Same as above	A-2 (0.4 *M* NaCl)	3
3	Calcium-lysed YP in 0.15 *M* NaCl + 0.05 *M* $CaCl_2$ (1% protein)	0.15 *M* NaCl + 0.05 *M* $CaCl_2$	A-3	3
4	Same as above except concentration of protein 0.25%	Same as above	A-3	3**
5	NaCl-lysed YP in 2.0 *M* NaCl + 0.05 *M* $CaCl_2$ (1% protein)	2.0 *M* NaCl + 0.05 *M* $CaCl_2$	A-3 (2.0 *M* NaCl)	5
6	Same as #3 except lysed YP was frozen before use	Same as #3	A-3	4
7	NaCl-lysed YP in 0.4 *M* NaCl (1% protein)	0.4 *M* NaCl	A-3 (0.4 *M* NaCl)	4
8	NaCl-lysed YP in 2.0 *M* NaCl (1% protein)	2.0 *M* NaCl	A-3 (2.0 *M* NaCl)	3
Control tubes: bovine serum albumin vs. antiserum; antigens vs. normal serum				0

* Rabbit antisera: A-1, anti-YP (lysed in 0.4 *M* NaCl). A-2, anti-"ghost" (from NaCl-lysed yolk suspension). A-3, anti-YP (lysed in 0.05 *M* $CaCl_2$).

** Better separation of bands than in tube #3.

DISCUSSION

1. *Solubility of yolk platelets and yolk "fractions"*

The studies reported here show that the major fraction of washed yolk platelet suspensions is readily soluble in lytic concentrations of NaCl or $CaCl_2$. If this solubility is taken as a criterion that the yolk proteins are in their native state, then it would appear that the homogenization-centrifugation procedure for isolating yolk platelets leaves them in a state similar to that *in ovo*. The remarkable stability of yolk platelet proteins is further demonstrated by platelet solubility in lytic concentrations of NaCl and $CaCl_2$, even after storage under refrigeration for many weeks. Detectable denaturation of yolk particles, either platelets or YC-fragments, seems to be limited to alterations of yolk surfaces in contact with homogenization or suspension media (Ringle and Gross, 1962). The stability of yolk proteins of isolated platelets increases the likelihood that studies on washed platelets will yield information pertinent to an understanding of yolk platelets *in ovo*.

The effects of solubilization of yolk by sodium and calcium salts are quite different, even apart from the permanent solubilization of yolk proteins by pro-

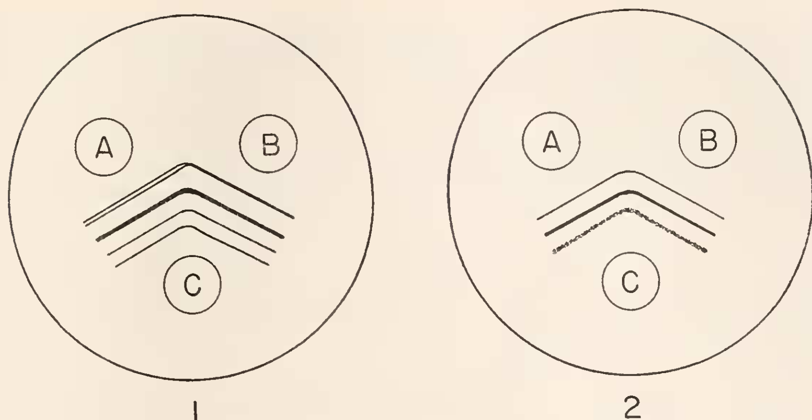

FIGURE 4. Ouchterlony Petri dish double diffusion method for the analysis of antigens in yolk and "ghost" material. Well contents: A, NaCl-solubilized YP; B, $CaCl_2$-solubilized YP; C, antiserum. Analysis 1: chicken antiserum (anti-YP_{ca}) in well C. Note five bands of antigen-antibody precipitate with YP_{na}, four bands with YP_{ca}. Analysis 2: rabbit antiserum (anti-"ghost") in well C. Controls (control serum in well C) negative for both analyses 1 and 2.

longed incubation with calcium ions. Differences in ionic strengths required for lysis, turbidity of the lysed yolk solutions, ease of denaturation, and kinetics of precipitation upon dilution all suggest a difference between the mechanisms of solubilization by NaCl and $CaCl_2$. Nevertheless, short exposure of solubilized yolk to calcium does not permanently alter its solubility characteristics, since the dilution precipitate of freshly calcium-lysed yolk can be dissolved in 0.4 *M* NaCl and will yield either YP or YC at the proper salt concentrations.

The different types of precipitate that form upon dilution of NaCl-lysed yolk (YP, YC, and WC) were at first thought to represent different protein fractions. However, since each of these "fractions" can be redissolved in 0.4 *M* NaCl and YP, YC and WC material produced from any one of these fractions, it is likely that they merely represent different physical states of the same yolk components. This apparent similarity is further verified by the electrophoretic analyses reported here.

Both YC and yolk platelets are dense materials, and YC was found to be identical in volume to the platelets from which it was derived. This similarity in volume indicates that the components of YC may be organized as in yolk platelets. The ability to transform viscous YC to a rigid, platelet-like material by a reduction in salt concentration also indicates a similar macromolecular packing in YC and platelets. Electron microscopy of YC-fragments and yolk platelets has shown a resemblance in the organization of these materials. A possible significance of this similarity between YC and platelets in relation to yolk synthesis has been suggested (Ringle and Gross, 1962).

2. *Extraction of lipids from* YP_{na}

Preliminary attempts to remove lipid from dried YP by extraction with hot ethanol: ether using a Soxhlet apparatus resulted in very poor yields of lipid.

Low lipid values with hot ethanol: ether have also been reported by Gross and Gilbert (1956). In view of the difficulty in extracting lipid from dried material, a method was used which enabled lipid to be extracted from fresh yolk in aqueous solution. The average value (10%) for lipid extracted with ethanol: ether solutions represents approximately 50% of the total yolk platelet lipid, based on yolk lipid values reported by Schjeide, Levi and Flickinger (1955). It is interesting that Macheboeuf (1953) was likewise able to remove only about half of the total serum lipids by a similar method of extraction with ethanol: ether.

Most or all of the carotenoids which impart the yellow color to amphibian yolk are extracted from YP_{na} by this method. It is likely, therefore, that the carotenoids are restricted to the more easily extracted lipid component of yolk lipoproteins. The preponderance of xanthophylls in YP agrees with the data reported by Fox (1953) for the carotenoids of frog ovaries.

Although removal of carotenoid-bearing lipid from yolk lipoproteins does alter the color and stickiness of YC made from defatted YP_{na} (YP_{df}), this removal of lipid does not noticeably alter the responses of yolk proteins to lytic concentrations of salts. The lytic properties of amphibian yolk seem, therefore, to reside with the protein or protein plus carotenoid-free lipid components.

3. *Electrophoresis*

The yolk fractions of *Rana pipiens* investigated here all showed two main electrophoretic peaks: a smaller, faster one and a larger, slower one (Fig. 3). This finding agrees with the results of other investigators on amphibian yolk. Flickinger and Nace (1952) found two electrophoretic fractions in the saline-soluble extract of mature *Rana temporaria* eggs. Barth and Barth (1954) also reported two fractions (a smaller, faster and a larger, slower one) in the KCl extract of *Rana pipiens* ovaries. They indicated the smaller fraction to be richer in phosphorus. Schjeide, Levi and Flickinger (1955) in studies on vitellin from washed platelets found one major component and a variable number of minor peaks preceding and trailing the main one. Two main yolk components have also been found by ultracentrifugation (Schjeide, Levi and Flickinger, 1955; Flickinger and Schjeide, 1957): a quantitatively smaller 6 S component rich in phosphorus and a larger 11 S component. Gross and Gilbert (1956) reported at least three fractions in *Rana pipiens* yolk by ultracentrifugation, with the main fraction showing a sedimentation value of 11 S. Thus both electrophoretic and ultracentrifugation studies have revealed two or more principal components in amphibian yolk.

Electrophoretic findings reported here concerning the occurrence of two peaks with similar mobilities and quantitative distribution support the idea that YP, YC, WC and YP_{df} are similar to one another. The slight asymmetry shown by the larger, slower electrophoretic peak of lipid-extracted YP_{df} indicates that this larger peak represents the principal lipid-bearing component of yolk.

4. *Phosphorus/nitrogen ratios*

As indicated in Table II, P/N ratios for "ghost" material are much higher than for whole yolk or yolk fractions (0.20–0.21 for "ghost" as compared with 0.13–0.14 for yolk platelets or unextracted soluble yolk). The higher P/N ratios of

"ghost" material support the conclusion that this saline-insoluble fraction is largely non-yolk in origin (Ringle and Gross, 1962).

Extraction of lipids from soluble yolk results in lower P/N ratios for the extracted yolk (0.093–0.12). It was not determined whether the removed phosphorus was contained in the ethanol: ether or the saline fraction after lipid extraction. In reference to the lower P/N ratios of lipid-extracted yolk, it is interesting that Panijel (1950) found that small yolk platelets contain less lipid and have lower P/N ratios than do large platelets.

There are indications that species differences exist for the P/N ratios of amphibian yolk. The 0.14 P/N ratio reported here for washed platelets of *Rana pipiens* is intermediate in value between the ratios reported by Panijel (1950) for platelet suspensions of *Rana fusca* (0.145) and *Rana esculenta* (0.125). Lower P/N ratios of 0.080 and 0.113, respectively, were calculated for the yolk of *Rana pipiens* from data of McClendon (1909) and Gross and Gilbert (1956).

Similar P/N ratios were found here for whole yolk platelet suspension and unextracted yolk fractions. Also, P/N ratios of platelets and YP remained constant, even after prolonged storage and dialysis. Panijel (1950), however, found that his platelet preparations lost up to 30% of their phosphoprotein phosphorus in 24 hours unless refrigerated at 0° C. or less in 15% sucrose. According to Panijel, 70.6% of the yolk phosphorus is from phosphoprotein, which would mean a loss of over 21% of the total yolk phosphorus. Such losses would markedly alter P/N ratios. Perhaps differences in the methods of preparation of washed platelets account for the differences in phosphorus lability.

5. *Nucleic acids*

Nucleic acid-P/N ratios reported here show a marked variability in the nucleic acid content of the various soluble and insoluble yolk platelet suspension fractions. This variability is related, at least in part, to the nature of the fraction involved. Thus, "ghost" preparations show a relatively high DNA-P content, which correlates with the cellular detrital nature of the bulk of this material. Nucleic acid-P/N ratios of "ghost" material are within the range of values obtained for a number of cells and tissues by other investigators (Leslie, 1955). There is also variability in nucleic acid content of soluble yolk prepared from different batches of washed yolk platelets. DNA-P/N and PNA-P/N ratios ranged, respectively, from 0–0.00095 and 0.00016–0.00072. Bieber, Spence and Hitchings (1959) have reported that both seasonal and dietary factors affect the DNA-content of the eggs of *Rana pipiens*.

Large quantities of DNA or DNA-like material, ranging from 0.012 to 1.23 μg. per egg or early embryo of amphibians, have been detected by a number of investigators (Kutsky, 1950; Hoff-Jørgensen and Zeuthen, 1952; Sze, 1953; Gregg and Løvtrup, 1955; Finamore and Volkin, 1958; Grant, 1958; Bieber, Spence and Hitchings, 1959). From the data of the studies reported here the theoretically possible contributions of DNA by yolk and "non-yolk" fractions were calculated from their DNA-P/N ratios (Table II). These calculations were based on DNA- and PNA-phosphorus values suggested by Schmidt and Thannhauser (1945) and the 112 μg. of yolk nitrogen per egg of *Rana pipiens* as reported by Gregg and Ballentine (1946). Although variable in amount, the calculated DNA from solu-

ble yolk ranged up to 1.08 μg. per egg, which is enough to account for the high DNA values of Kutsky (1950) and Sze (1953). In addition the "non-yolk" homogenate supernatant could itself supply a significant amount of DNA to the egg (0.18 μg.).

PNA-P values for the various yolk samples also showed a fairly marked variation, and some were much lower than 0.01, a ratio calculated for platelets of *Rana esculenta* from data of Panijel (1950). The higher PNA-P/N ratio for "non-yolk" homogenate supernatant is probably related to the presence of microsomes.

Unfortunately there are many factors that make it difficult to assign specific nucleic acid values to yolk and yolk fractions from homogenized eggs. The presence of "ghost" material in platelet suspensions introduces the possibility that some nucleoprotein is extracted from "ghost" nuclei during platelet lysis. However, the small quantity (0.3% or less) of "ghost" nitrogen in platelet suspensions and the short time involved in lysis would not indicate such an origin for the DNA of YP. The determination of normal nucleic acid values for yolk is also complicated by the prolonged washing procedures necessary for the preparation of relatively clean suspensions. Although the solubility of DNA-proteins is not marked at the low (0.1 *M*) NaCl concentration used for washing and storage of platelets (Frick, 1949), some nucleoprotein material could be lost during washing and storage. Possible surface adsorption of nucleoproteins during homogenization also complicates interpretation of results. Nevertheless, the occurrence of DNA in washed platelet suspensions and solubilized yolk corroborates the weakly positive Feulgen reaction shown by yolk platelets fixed *in ovo* (Hibbard, 1928; Brachet, 1950; Ringle and Gross, 1962).

6. *Antigenic analyses*

The results of double diffusion experiments reported here show that soluble yolk possesses a minimum of 5 antigens. This finding is compatible with the results of Cooper (1950) who reported 5–7 antigens for the eggs of *Rana pipiens*. Since she was dealing with eggs or egg extracts, a greater number of antigens might be expected than in washed yolk suspensions, which would account for the occurrence of as many as 7 antigens. Using calcium-lysed yolk prepared from washed yolk platelets to evoke antibody production in rabbits, Flickinger and Rounds (1956) found a maximum of 5 antigens in both calcium- and sodium-lysed yolk. They found that both types of lysed yolk yield the same band precipitate pattern in agar, thus indicating the antigenic similarity of these lysed preparations. The results of the Petri dish method reported here also indicate a similarity for the antigens of calcium- and sodium-lysed yolk, although two of the bands of sodium-lysed yolk formed only a single band with calcium-lysed yolk (Fig. 4).

Differences in the number of bands of precipitate formed during double diffusion does not necessarily indicate differences in antigen number. The effects of varying salt and protein concentrations on the number of bands formed by reacting antigen-antibody systems are evident from the results reported in Table IV. In addition to different numbers of bands, marked differences were sometimes noted in the appearance of bands from tubes containing the same antigens and antibodies (but different salts or salt concentrations). Thus, although tube #8 (Table IV) gave only three bands as opposed to five bands in tube #5, one of the three bands

in tube #8 was much broader and presumably contained more precipitate than any single band in the other tube. It is suggested that the more prominent band contained precipitate which formed several bands in the presence of added 0.005 *M* $CaCl_2$. Although differences in tube contents caused differences in band formation, duplicate tubes always gave similar results. The differences in number and appearance of bands under different experimental conditions demonstrate that a variety of results can be obtained from the same antigens and antisera, depending upon the other reagents used. The effects of reagent concentration on band formation in agar have been discussed by Oudin (1952), Preer (1956), Preer and Telfer (1957) and Wilson (1958).

A number of different salt combinations and concentrations were tested because soluble yolk forms a precipitate upon diffusion into agar (at the agar-yolk interface), even when the agar contains 0.4 *M* NaCl. By increasing the NaCl content of agar to 2.0 *M* it is possible to eliminate this non-specific precipitate. Flickinger and Rounds (1956) also found this type of precipitate and that it could be prevented by adding 10% NaCl to the agar.

Freezing yolk prior to its use against antiserum promotes the formation of a larger number of bands than form from non-frozen yolk (Table IV, tubes #3 and #6). Also, bands from frozen yolk appear more rapidly than do bands from non-frozen yolk. In view of the damaging effects of freezing on lipoproteins (Lovelock, 1957), the change in band formation caused by freezing suggests that antigenic substances are thereby made more free to escape from or move through the non-specific yolk-agar precipitate.

Chicken antisera were prepared to determine if more precipitate bands would result than with rabbit antisera in agar containing 2.0 *M* NaCl. Goodman and Wolfe (1952) reported that maximum precipitation occurs with chicken antisera at 8–12% NaCl, whereas salt concentrations higher than 0.15 *M* NaCl reduce the amount of specific precipitate with rabbit antisera (Kabat and Mayer, 1948). The results with chicken anti-yolk antisera do not, however, reveal more than the maximum number of 5 bands found with rabbit antisera (Fig. 4).

Injection of suspended washed "ghost" material into rabbits causes the production of antibodies which react with at least three of the antigens present in soluble yolk (Table IV and Fig. 4). "Ghost" antigenicity could be caused by the normal presence of yolk antigens in the cellular material, by adsorption of soluble yolk during lysis, or by the insoluble ghosts (surface residues) of lysed platelets.

Summary

1. Washed yolk platelet suspensions (prepared from ovarian eggs of *Rana pipiens*) lyse in the proper concentrations of NaCl or $CaCl_2$, resulting in solubilized yolk (YP_{na} or YP_{ca}) and an insoluble "ghost" residue. Different precipitation effects result with NaCl and $CaCl_2$ at lowered salt concentrations.

2. A compact precipitate (YC) resembling yolk platelets in density and volume results from a reduction of the salt concentration of YP_{na} to 0.28–0.30 *M* NaCl. A further reduction in salinity transforms YC from a plastic to a rigid material.

3. About 10% of the dry weight of soluble yolk is readily extracted with 9.6% ethanol: ether without affecting the solubility properties of the yolk proteins. The extracted lipids contain all or most of the yolk carotenoids, primarily xanthophylls.

4. Electrophoretic analyses show yolk fractions precipitated from YP_{na} by dilution (YP, YC, and WC) to be similar. Lipid-extracted yolk (YP_{df}) also gave a similar electrophoretic pattern.

5. Differences between the phosphorus/nitrogen ratios of whole yolk suspension and YP_{na} (0.14) and "ghost" material (0.20–0.21) support the conclusion that "ghost" material is largely non-yolk in origin.

6. Both DNA-P and PNA-P are detected in washed yolk platelet suspension and in soluble yolk, but the quantity found varies among different batches of yolk. The amount of DNA-P in some preparations is sufficient to account for the excess DNA/egg reported by previous investigators.

7. A minimum of 5 antigens are found for solubilized yolk, using both rabbit and chicken antisera. "Ghost" material shows three antigens in common with soluble yolk.

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